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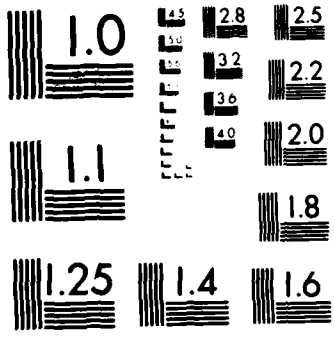

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Tissue Culture and In Vivo Modelling of Corneal Opacification and Ocular Injuries Induced by Millimeter Waves

Annual Summary Report

DR. JOHN R. TREVITHICK

June 1982

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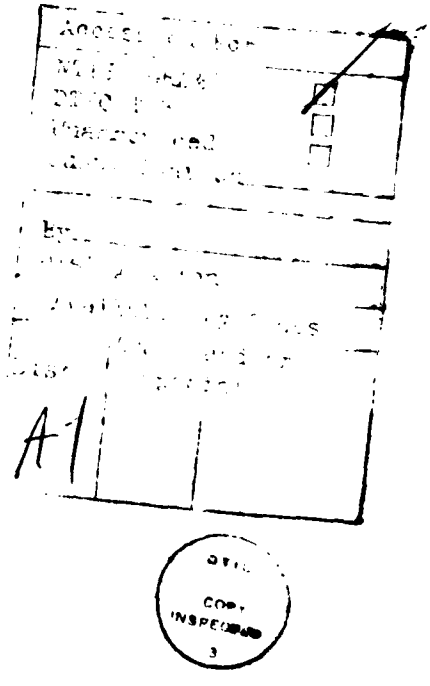
FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



SUMMARY

The eventual aims of these experiments are to use intact corneas incubated in vitro and corneas of rats exposed in vivo to high energy pulsed millimeter waves to study the development of corneal damage. Such experiments are expected (1) to establish conditions for corneal damage and (2) to elucidate the mechanisms by which the damage occurs.

If intact corneal explants are cultured in tissue culture dishes in medium at 35.5 C, continuing outgrowth of the epithelial cells occurs from the edge of the explants during a period of at least two weeks (reported in 1981). Exposure for shorter periods of such cultured corneas to elevated temperatures and vitamin A followed by fixation for light microscopy (SEM) revealed progressive cellular damage as the temperature increased and some vitamin A uptake which is being studied further, by biochemical techniques.

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INTRODUCTION

Although millimeter wave radars are now strategically important, only one study of the effect of millimeter waves on the cornea has been reported (Rosenthal et al, 1975). This study did not use the pulsed mode of millimeter waves which is commonly in use in such radars. Because we have discovered apparent differences between similar doses of pulsed and CW microwaves (Stewart-DeHaan et al, 1980) in preliminary experiments and because we have succeeded in separating the effect of heating from the effects due to the electromagnetic field, for microwaves, we wished to devise a similar system for irradiation of the cornea in vitro which would offer similar advantages for the study of millimeter wave damage to the cornea.

The first step in these experiments was to devise appropriate media and conditions for the tissue culture of corneas, to be used for the experiments investigating their exposure to millimeter waves. The second step, also described in last year's report, was to incubate the cultured corneas at different elevated temperatures in order to investigate the effect of incubation at elevated temperature on the cornea. The third stage, reported here, was to test the effect of exposing incubated corneas to non-ionizing radiation, for which we selected ultraviolet as convenient and accessible in our laboratory, since the appropriate millimeter wave irradiation apparatus is not operating yet.

MATERIALS AND METHODS

Corneal Culture

Rabbit eyes are obtained at the nearby slaughterhouse and brought to the laboratory at room temperature for short periods of time (less than half an hour). They are rinsed several times in sterile phosphate-buffered saline (PBS) so as to remove most bacteria. Furthermore, bacterial and fungal growth is inhibited by inclusion of antibiotics (penicillin, streptomycin, and fungazone) in the medium 199 used for the incubations.

Corneas are removed by trimming around the edge of the cornea using iris scissors, and placed right side up on a small triangular boat in a culture dish with medium 199, as described by Buck (1979), with the upper surface exposed to the air.

Phase Contrast and Interference Phase Microscopy

Corneas on boats in culture dishes are examined by an inverted microscope (Leitz Diavert) equipped with optics which permit either interference phase or tradiational phase contrast microscopy. For convenience, usually the more customary phase contrast optics were used in these studies.

Embedding in Water-Soluble Embedding (WSE) Media, and Thin Sectioning

We have now developed this technique for small pieces of cornea. The technique provbides for improved resolution of microscopic details (as compared to conventional wax-embedded) in plastic formed from water-soluble methacylate. Such sections may be stained by conventional techniques (toluidine blue or hematoxylin and eosin) or subjected to radioautography.

Scanning Electron Microscopy

In addition to conventional SEM, described in Figures 2-4, Dr. Creighton has revealed the structure of cytoplasmic matrix and nucleus and the cytoskeleton of corneal cells by removing the overlapping cell membrane adhering to Scotch "Magic" brand tape pressed on the surface of critical-point-dried corneas.

Biochemical Techniques

Techniques for analysis of protein, glutathione, Vitamin C, malonaldehyde, ATP and adenylate energy charge, lipid analysis and Vitamin E were described in our grant application and progress report in 1981 (Grant No. DAMD-17-80-G-9480). Following the recent development of techniques which appeared to satisfactorily meet the criteria for morphological integrity of corneal cells, we are now investigating the system with respect to these biochemical parameters with a view to optimizing them, and to evaluating the changes, both morphological and biochemical which occur in the cornea when it is subjected to short periods of elevated temperature.

Results

In our 1981 report we discussed our progress in (1) tissue culturing of corneal epithelial cells, (2) observation of the cytoskeleton by high voltage electron microscopy, or SEM, after removal of the cell membrane using Scotch "Magic" tape, (3) observations of the damage caused by elevated temperatures on the corneal epithelium. The relevant sections from that report are summarized below:

a) Tissue Culture Medium Which Supports Outgrowth of Corneal Epithelial Cells

Several tissue culture media were tested for ability to support outgrowth of corneal epithelial cells, in a preliminary screening experiment. The culture conditions which appeared to give optimum conditions for corneal maintenance were described. In these experiments, the corneal epithelial cells migrated and grew from the original explant over a period of several weeks. Although it seems that these conditions are suitable for corneal culture, further study of the relative role of serum in the medium seems necessary since it has recently been suggested by Van Horn's group to result in increased vacuolation of the epithelial cells (Tierney et al, 1981).

b) Cytoskeletal Observation

Although further stereoscopic investigations of the cytoskeleton by HVEM are planned, a breakthrough in examination of the cytoskeleton was made, so that evaluation of cytoskeletal structural changes by a technique involving scanning electron microscopy now seems possible. Although further work will be required to establish definitively how this technique may be applied and interpreted, removal of the cell membrane by simple adhesion to Scotch brand "Magic" tape provides a quick and easy technique for visualizing the internal cell contents and cytoskeleton.

Discussions: Cytoskeletal Observation

The Scotch tape technique for cytoskeletal visualization has the advantages of permitting the fixed cell contents and nuclear contents of the fixed cell to be observed in a "native" state. Several other techniques which have been used for removal of cytoplasmic membranes involve the solubilization of the cell membrane by detergent solutions. Although these techniques have also permitted visualization of cytoplasmic contents, the exposed cytoplasm has also been subjected to the detergents, which may give rise to artefacts and complicate interpretation of the results.

c) Effects of Elevated Temperature on the Corneal Epithelium

Elevated temperatures seemed to cause striking changes in morphology of the corneal epithelium. At 37°C, the junctions between corneal epithelial cells appeared as a ridge and the cells surfaces were covered by short stubby cylindrical microvilli. At 39°C, the junction between cells became a trough, possibly due to cell swelling, and microvilli were disorganised, elongated and intertwined cylinders. At 42°C, the epithelial cells tore apart and some were missing from the surface, leaving devided areas; microvilli were sparse and appeared only as small bumps on the surface. After exposure to higher temperatures, (45°C and 50°C) a progression appeared to occur involving cells curling up and apparently dying with complete loss of microvillae, resulting in denudation of large areas of such corneas.

NEW RESULTS

In the current year, work has proceeded on culturing intact corneas *in vitro* under conditions closely approximating those found *in vivo*. The lower (concave) corneal surface is immersed in tissue culture medium, while the upper convex surface as *in vivo*, is in contact with air (Fig. 1). The apparatus used for these experiments will require some modifications to eliminate metal parts which would absorb millimeter waves. To test the effect of a similar stress, ultraviolet irradiation, we exposed corneas suspended as shown to ultraviolet irradiation$_2$(254 nm) using a 15W General Electric G15T8 bulb, which delivers 104 ergs/mm^2/sec to the tissue. Following the irradiation, the corneas were incubated for 24 hr (Fig. 3) or 48 hr (Fig. 4). Normal corneal epithelial cells showed prominent microvilli, a normal feature, (Fig. 2) after 48 hr incubation, but appeared to be somewhat swollen, since the corneal cell borders were slightly depressed in level by comparison to the remainder of the cell surface. Following UV irradiation, progressive damage to the corneal epithelium appears to occur as the length of the incubation increases. At the end of 24 hr incubation (Fig. 3), the cell surfaces were rather smooth, with microvillae truncated or lost, while at the cell borders, which appeared irregular and jagged, with many long processes extending at cell borders. After 48 hr of incubation many epithelial cells were lost (Fig. 4A,B), and the microvillae have become grossly swollen and distorted, leading to a very disorganized fibrillar surface (Fig. 4C,D). If fluorescein staining were performed the denuded areas would stain intensely with rose bengal or fluorescein. Some correlation with such staining will be necessary.

DISCUSSION

These results establish conditions for successfully culturing isolated rabbit corneas. They have extended our previous studies in which corneas were cultured submerged in tissue culture to a more natural system in which corneas are cultured with the apical epithelium in air and the endothelium submerged in tissue culture medium. They have tested the effect of a stress similar to millimeter waves, on such cultured corneas ultraviolet light. These studies indicate that the SEM appears to provide adequate experimental information.

It may be necessary to perform some studies of the cornea using a standard ophthelmic microscope; this is especially useful in preliminary experiments for routinely following by photographic techniques the fluorescein or rose bengal staining of the cornea, and by interferometry, for necessary changes in corneal thickness.

Extending the present techniques to millimeter wave studies may be somewhat simplified by the new technique of cryological-SEM-EDX; this technique is expected to have the following advantages:

1) Because specimens are frozen it should be possible to determine corneal thickness in cross sections (allowances for the expansion of water on freezing will have to be made). This could complement our proposed use of the ophthalmic microscope for some studies (see 1982 application for grant). Such measurements would be invasive, and for one time point, and not continuous as possible for the ophthalmic microscope.

2) Because it is expected to be possible to monitor ionic concentrations in addition to observing the appearance by SEM of such sections, it should be convenient using the in vitro system to test parametrically several regimens. These might involve changes in irradiation conditions temperature, and medium supplementary to tests in vivo for which such variations are not easily possible.

It will be necessary to develop an appropriately modified chamber for the actual in vitro radiation, so that the following are satisfactory (1) control of temperature, flow rate and humidity of air in contact with upper corneal surface, (2) control of temperature and flow rate of circulating medium in contact with lower corneal surace, (3) transfer of the cornea on its grid to standard incubation medium after a period of irradiation. Should in vivo irradiation be attempted, similarly modifications will be necessary to permit control of corneal surface temperatures in live animals by a flow of mast air on the corneal apical surface.

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FIGURE LEGENDS

Figure 1. Photograph and diagram illustrating the wire mesh supported by teflon cylinders, with cornea being incubated.

Figures 2-4. Corneas were incubated in Medium 199 at 35.5°C according to Buck in a CO_2 incubator for 24-48 hr, fixed in Karnovsky's fixative for 48 hr at 4°C, placed in 0.1 M cacodylate pH 7.2 for 48 hr dehydrated in an ethanol series of CO_2, critical-point dried, sputter coated and viewed by scanning electron microscopy (SEM).

Figure 2. Control: normal rabbit cornea. Note regular surface structure with numerous short microvillae. Cells have swollen slightly leaving depressed intercellular borders.

Figures 3-4. Scanning electron microscopy of corneas exposed to ultraviolet irradiation (6 cm from an ultraviolet light source (General Electric G15T8 (15 watt) wavelength 254 nm); 104 ergs/mm^2/sec for 5 min, at a distance of 6 cm from the light) prior to incubation in medium 199 prior to fixation and critical point drying.

Figure 3. After 24 hr incubation following UV treatment surface is smoother, with microvillae truncated or lost, while cells appear to be contracting leaving irregular long processes extended between intercellular borders.

Figure 4. After 48 hr of incubation, many cells are lost (see A and B) or showed the large swollen processes and irregular fibrillae on the cell surfaces (C and D).

Figure 1

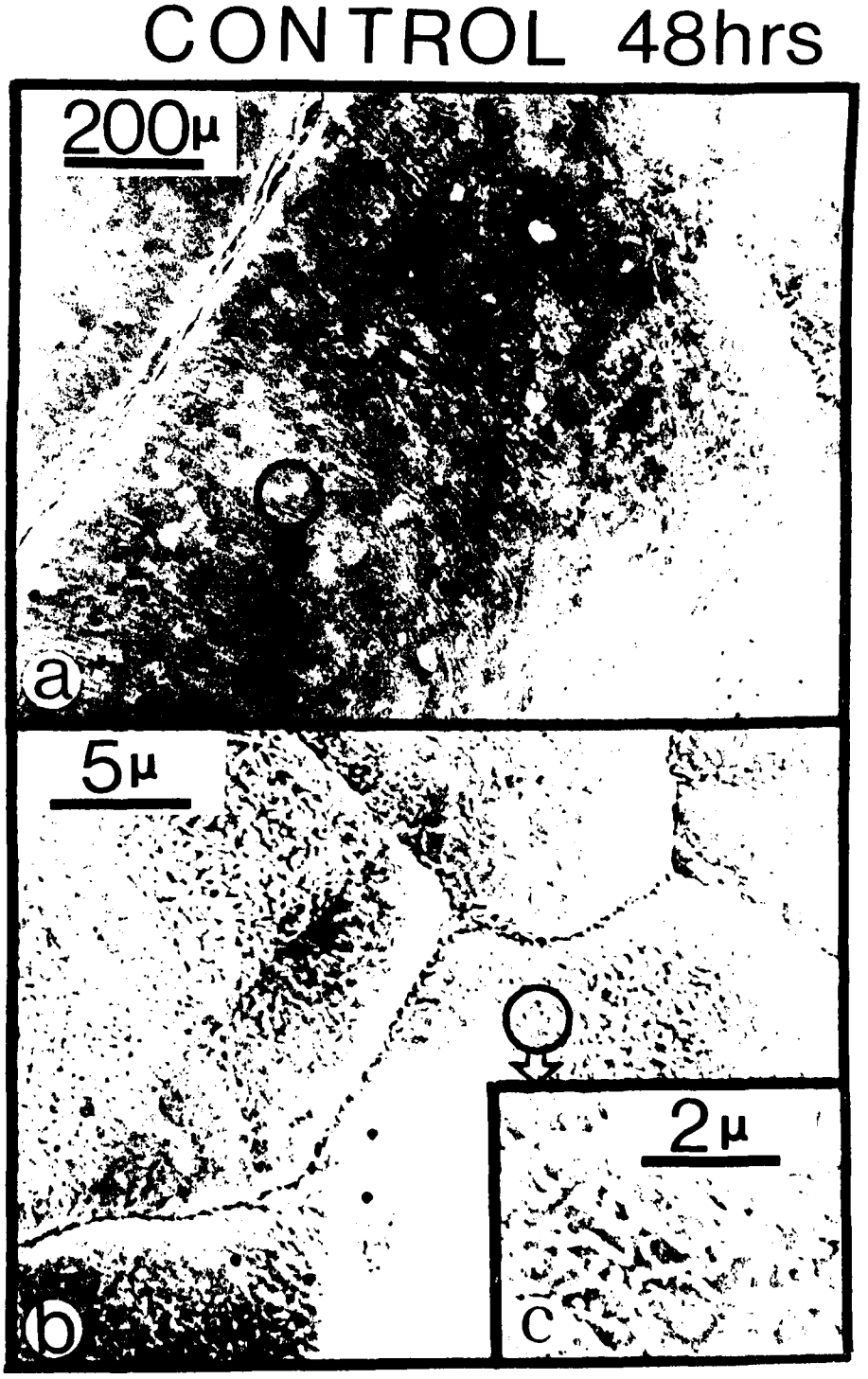

Figure 2

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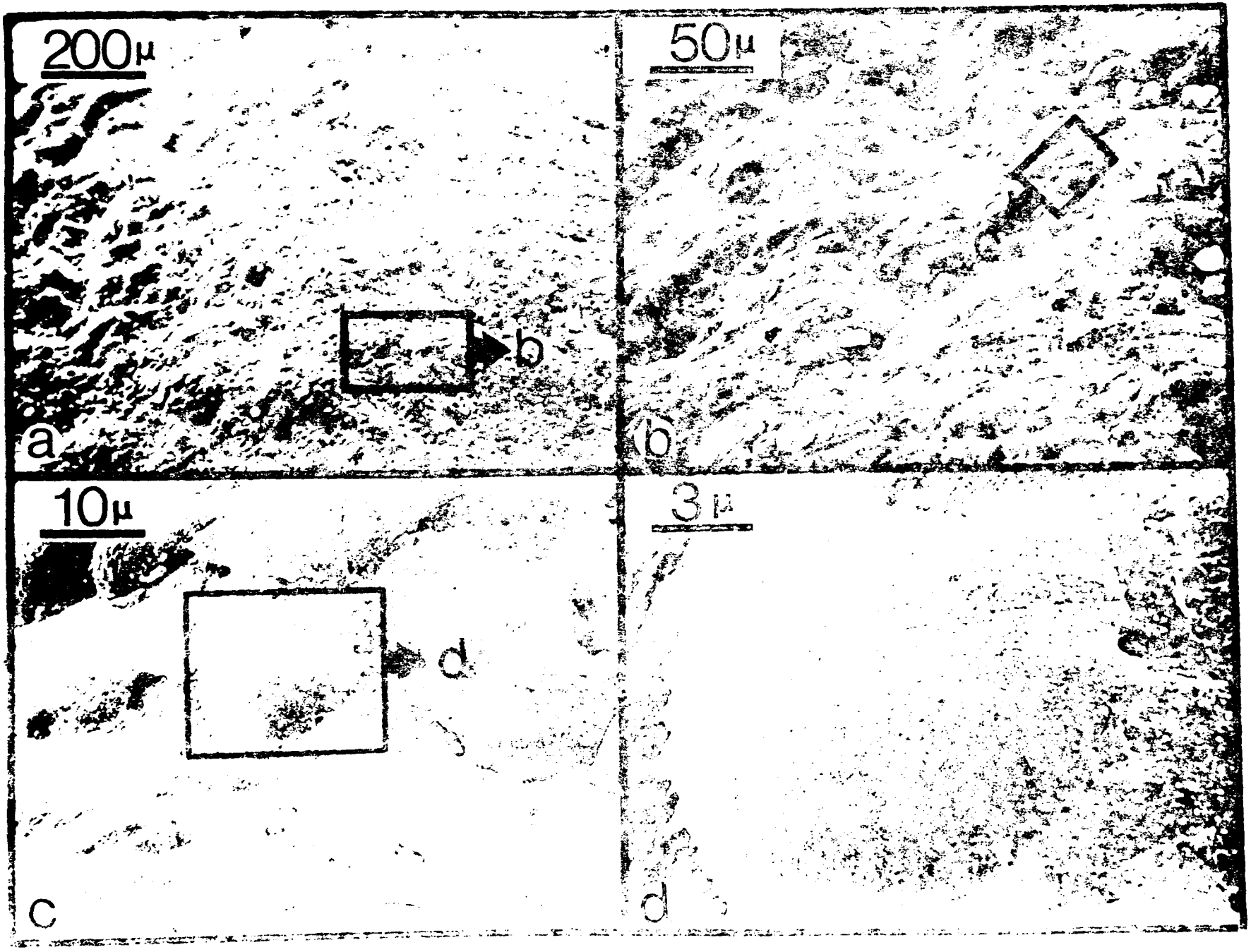
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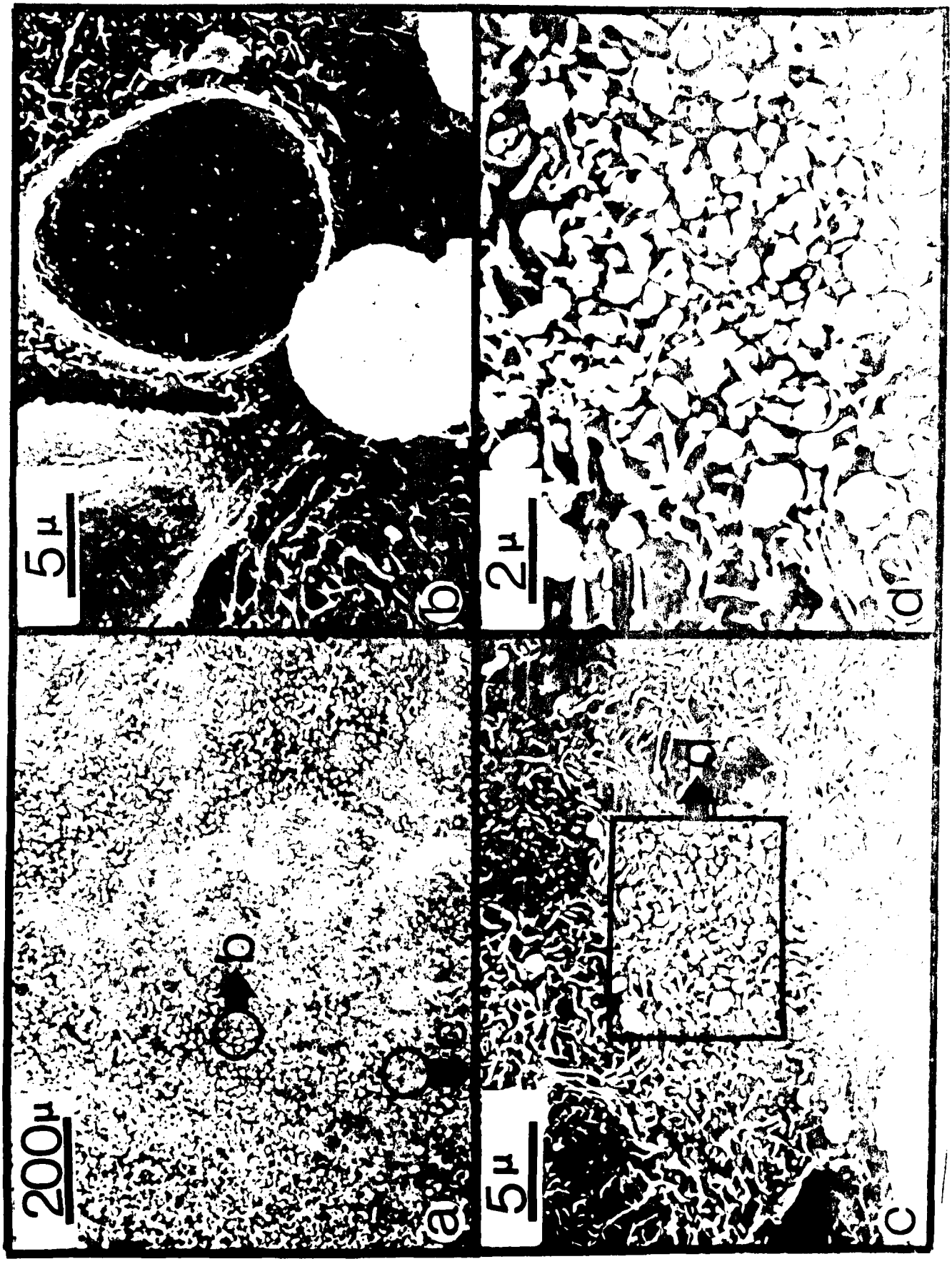

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